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Inhibitory function of adapter-related protein complex 2 alpha 1 subunit in the process of nuclear translocation of human immunodeficiency virus type 1 genome

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Abstract

The transfection of human cells with siRNA against adapter-related protein complex 2 alpha 1 subunit (AP2α) was revealed to significantly up-regulate the replication of human immunodeficiency virus type 1 (HIV-1). This effect was confirmed by cell infection with vesicular stomatitis virus G protein-pseudotyped HIV-1 as well as CXCR4-tropic and CCR5-tropic HIV-1. Viral adsorption, viral entry and reverse transcription processes were not affected by cell transfection with siRNA against AP2α. In contrast, viral nuclear translocation as well as the integration process was significantly up-regulated in cells transfected with siRNA against AP2α. Confocal fluorescence microscopy revealed that a subpopulation of AP2α was not only localized in the cytoplasm but was also partly co-localized with lamin B, importin β and Nup153, implying that AP2α negatively regulates HIV-1 replication in the process of nuclear translocation of viral DNA in the cytoplasm or the perinuclear region. We propose that AP2α may be a novel target for disrupting HIV-1 replication in the early stage of the viral life cycle.
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Introduction

Human immunodeficiency virus type 1 (HIV-1) is a causative agent of acquired immune deficiency syndrome (AIDS). HIV-1 replication is governed by a complex regulatory mechanism, and many host factors are involved either positively or negatively in HIV-1 replication (Rice and Kimata, 2006; Zheng et al., 2005). Some such host factors were found to be determinants of cell tropism and/or the host range of HIV-1 (Cullen, 2003; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996; Sokolskaja and Luban, 2006; Stremlau et al., 2004; Wei et al., 1998; Zheng et al., 2003). Although many host factors have already been identified, the regulatory mechanism of the HIV-1 life cycle is still not fully understood.

RNA interference (RNAi) has been found as a highly effective and widely used methodology for the suppression of specific gene expression in eukaryotic cells. Small interfering RNA (siRNA), comprised of a duplex of two 21-mer RNAs with 19 complimentary nucleotides and 3′ terminal 2 non-complementary nucleotides, can induce the RNAi-mediated specific suppression of target genes in eukaryotic cells (Elbashir et al.,

2001). In order to find the novel host factors affecting HIV-1 replication, we previously studied the level of HIV-1 replication in cells transfected with a series of siRNAs directed against cellular genes selected as functional molecules to be involved in intracellular signal transduction pathways, intracellular transportation processes and the cytoskeletal system (siRNA mini-library) (Kameoka et al., 2007; Ui-Tei et al., 2004). We found that the levels of the single replication cycle of luciferase reporter HIV-1 were significantly enhanced in cells transfected with siRNAs against adapter-related protein complex 2 (AP-2) alpha 1 subunit (AP2α), ADP-ribosylation factor 6, Axin1, dual specificity phosphatase 1, Janus kinase 1 and partitioning defective 6 homolog alpha (Kameoka et al., 2007), suggesting that those cellular proteins may negatively regulate HIV-1 replication under normal cell culture conditions. Among them, siRNA against AP2α most effectively enhanced HIV-1 replication (Kameoka et al., 2007).

AP2α is a major component of AP-2 that is known to regulate receptor-mediated, clathrin-dependent endocytosis of plasma membrane proteins (Connor and Schmid, 2003; Nakatsu and Ohno, 2003; Robinson, 2004). Several reports suggest that AP-2 plays roles in HIV-1 replication (Batonick et al., 2005; Boge et al., 1998; Daecke et al., 2005). AP-2 was shown to play negative roles in the late phase of the viral life cycle (Batonick et al., 2005; Boge et al., 1998), while it was also shown to play a role in alternative, endocytosis-mediated HIV-1 entry into cells (Daecke et al., 2005). In this study, we show that siRNA against AP2α enhanced the level of nuclear viral DNA as well as of integrated proviral DNA, while viral adsorption, viral entry and reverse transcription (RT) processes were not notably affected by siRNA against AP2α. These results suggest that AP2α plays a role(s) in HIV-1 replication in the process of nuclear translocation of viral DNA, but not in earlier process such as viral entry in the plasma membrane. We also revealed that a subpopulation of AP2α was not only localized in the cytoplasm, but also partly co-localized with proteins functioning in the perinuclear region, implying that AP2α may play a negative role in HIV-1 replication in the cytoplasm or the perinuclear region.

Results

siRNA directed against AP2α significantly enhances HIV-1 replication

Previously, we studied the replication level of vesicular stomatitis virus G protein (VSVG)-pseudotyped, luciferase reporter HIV-1 (HIV-1/VSVG) in J111 cells transfected with a series of siRNAs directed against cellular genes (siRNA mini-library). Among 257 siRNAs, siRNA directed against AP2α most significantly enhanced the level of a single replication cycle of luciferase reporter HIV-1 (Kameoka et al., 2007). In this report, we further studied the regulatory mechanism of AP2α in HIV-1 replication. First, we verified the results of screening tests using the siRNA against AP2α (AP2α siRNA1) that was equivalent to the one included in siRNA mini-library. J111 cells were transfected with AP2α siRNA1 or control siRNAs. The cells were

then infected with HIV-1/VSVG. In addition, we examined the replication of CXCR4-tropic (X4) HIV-1 (HIV-1/X4) and CCR5-tropic (R5) HIV-1 (HIV-1/R5), as well as of HIV-1/VSVG, in MAGIC5A cells transfected with AP2α siRNA1. Using these reporter viruses bearing the luciferase gene in place of the viral *nef* gene, the efficiency of the early phase of the HIV-1 replication cycle, including viral entry, RT, nuclear translocation, integration, RNA transcription and protein translation, could be monitored by measuring luciferase activity in infected cells (Chen et al., 1994; Connor et al., 1995). The results showed that the replication of HIV-1/VSVG (Fig. 1B, left and right upper panels), as well as HIV-1/X4 and HIV-1/R5 (Fig. 1B, right upper panel), was strongly up-regulated by cell transfection with AP2α siRNA1. The replication of three reporter viruses which enter cells by means of interaction with different transmembrane molecules seemed to be likewise enhanced by cell transfection with
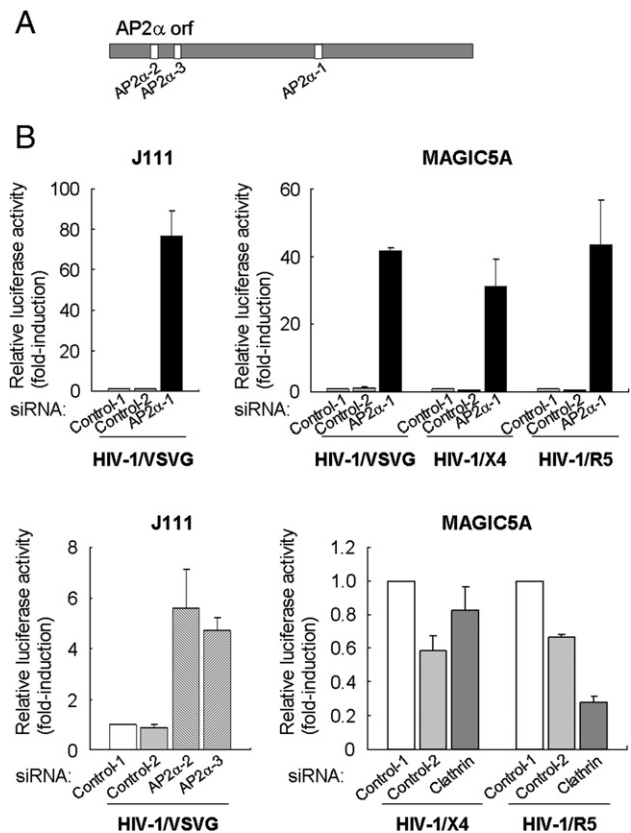

Fig. 1. siRNA against AP2α, but not against clathrin, significantly enhances HIV-1 replication in the early stage of the viral life cycle. (A) Schematic illustration of the target position of three siRNAs in the open reading frame (orf) of AP2α gene. (B) J111 (left upper and lower panels) or MAGIC5A cells (right upper and lower panels) were transfected with AP2α siRNA1 (AP2α-1: solid bar), AP2α siRNA2 (AP2α-2: left hatched bar), AP2α siRNA3 (AP2α-3: right hatched bar), clathrin siRNA (clathrin: dark gray bar), control (non-silencing) siRNA (Control-1: open bar) or FLJ10847 siRNA (Control-2: light gray bars), as indicated. Forty-eight hours after transfection, the cells were infected with HIV-1/VSVG, HIV-1/X4 or HIV-1/R5, as indicated below the panels. Twenty-four hours after infection, luciferase activity in infected cells was measured. Data are shown as fold induction relative to luciferase activity in cells transfected with control siRNA. Data are presented as the means and standard deviations (error bars) of more than three independent experiments.
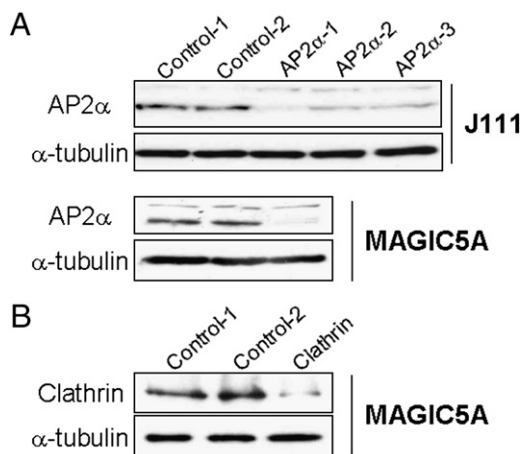
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Fig. 2. siRNAs against AP2α and clathrin specifically suppressed the expression of their target gene products. J111 and MAGIC5A cells, as indicated beside the panels, were transfected with indicated siRNA. Forty-eight hours after transfection, the cell lysate was prepared and subjected to immunoblot analysis using anti-AP2α (A), clathrin (B) or α-tubulin (A and B) antibodies, as described in Materials and methods.

AP2α siRNA1; therefore, we consider that AP2α plays a negative role in HIV-1 replication after the entry process of the viral life cycle under normal cell culture conditions. To confirm the specific RNAi effect of siRNA on AP2α gene expression, we tested three siRNAs targeting different position in the AP2α gene (Fig. 1A). The results showed that three siRNAs against AP2α exhibited similar tendencies of the enhancing effect on HIV-1/VSVG replication (Fig. 1B, left upper and lower panels) and of the suppressing effect on AP2α expression (Fig. 2A). Among three siRNAs against AP2α, AP2α siRNA1 was most effective on AP2α expression (Fig. 2A) as well as on HIV-1 replication (Fig. 1B, compare left upper and lower panels). AP2α siRNA1 was designed as highly effective and specific siRNA based on the novel technique described previously (Ui-Tei et al., 2004), and therefore AP2α siRNA1 might be more effective compared with AP2α siRNA2 or 3. We used AP2α siRNA1 for the subsequent experiments described below.

Clathrin-dependent endocytosis is not involved in AP2α siRNA-induced up-regulation of HIV-1 replication

AP2α is a major subunit of AP-2 (Nakatsu and Ohno, 2003). Since AP-2 is known to play a role in the clathrin-dependent endocytosis mechanism, this type of endocytosis is possibly involved in the AP2α siRNA-induced up-regulation of HIV-1 replication observed. To examine this possibility, we studied the replication of HIV-1/X4 and HIV-1/R5 in MAGIC5A cells transfected with siRNA directed against clathrin. The siRNA against clathrin, which inhibits clathrin-dependent endocytosis (Motley et al., 2003), effectively suppressed clathrin expression (Fig. 2B) and HIV-1 Nef-dependent CD4 down-regulation (Supplementary Fig. S1), whereas the siRNA against AP2α weakly suppressed Nef-dependent CD4 down-regulation (Supplementary Fig. S1), consistently with a previous report (Chaudhuri et al., 2007). The results showed that the level of replication of HIV-1/X4 in clathrin siRNA-transfected cells was comparable

to that in cells transfected with control siRNAs (Fig. 1B, right lower panel). In addition, the replication of HIV-1/R5 was rather suppressed by transfecting cells with siRNA against clathrin (Fig. 1B, right lower panel). Namely, the suppression of clathrin-dependent endocytosis does not enhance HIV-1 replication. Based on these results, we can conclude that siRNAs against AP2α enhance HIV-1 replication independently of the clathrin-dependent endocytosis mechanism.

siRNA against AP2α does not affect viral adsorption and viral entry processes of HIV-1 replication

We next studied the levels of viral adsorption and viral entry processes of HIV-1 by measuring the amount of p24 antigen that binds to and penetrates into cells, respectively. Cells were transfected with siRNA against AP2α and then infected with HIV-1. After the washing process, cell lysates were prepared and the amount of p24 antigen in the samples was measured. The results showed that the levels of viral adsorption (Fig. 3A, left panel) and viral entry (Fig. 3B, left panel) of HIV-1/VSVG were comparable among cells transfected with siRNA against AP2α or control siRNAs. In addition, the levels of viral adsorption (Fig. 3A, right panel) and viral entry (Fig. 3B, right panel) of HIV-1/X4 and HIV-1/R5 were rather slightly decreased in AP2α siRNA-transfected
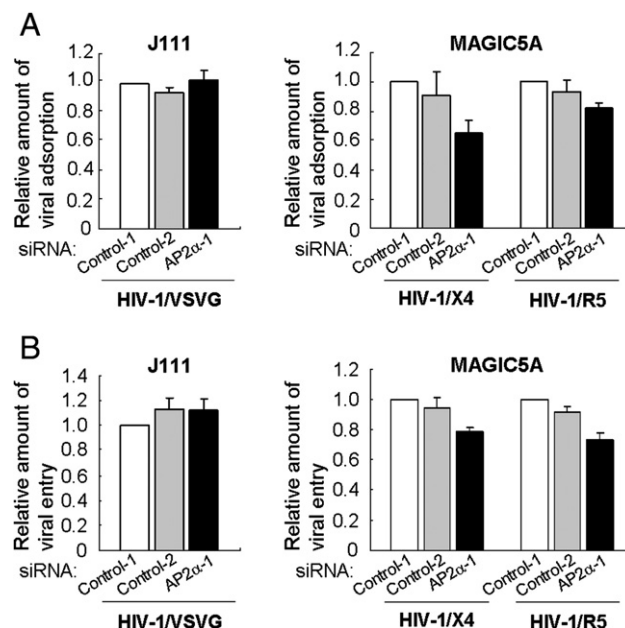

Fig. 3. siRNA against AP2α does not affect viral adsorption or viral entry into cells. J111 (left panels) or MAGIC5A cells (right panels) were transfected with AP2α siRNA1 (AP2α-1: solid bar), control (non-silencing) siRNA (Control-1: open bar) or FLJ10847 siRNA (Control-2: light gray bar). Forty-eight hours after transfection, the cells were infected with HIV-1/VSVG, HIV-1/X4 or HIV-1/R5, as indicated below the panels. The levels of viral adsorption and viral entry were examined, as described in Materials and methods. As control experiments, cells were incubated with Env-deficient viral particles, and the levels of Env-independent, non-specific adsorption and viral entry were estimated. The relative amounts of Env-dependent viral adsorption (A) and viral entry (B) were then calculated by subtracting the amounts of such non-specific viral adsorption and entry. Data are presented as the means and standard deviations (error bars) of three independent experiments.
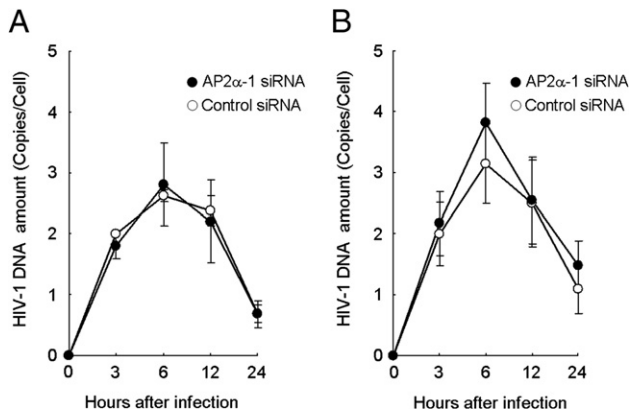
Fig. 4. HIV-1 RT process is not affected by cell transfection with siRNA against AP2α. J111 cells were transfected with AP2α siRNA1 (AP2α-1: solid circle) or control (non-silencing) siRNA (open circle). Forty-eight hours after transfection, the cells were infected with DNase I-treated HIV-1/VSVG (40 ng of p24). Cellular DNA was extracted at 3, 6, 12 and 24 h after infection, and subjected to real-time PCR using the primers/probe sets for detecting early (A) or late (B) RT products. The copy number of HIV-1 DNA per cell was calculated as described in Materials and methods. Data are presented as the means and standard deviations (error bars) of more than three independent experiments.

cells compared with control cells. Thus, siRNA against AP2α did not up-regulate viral adsorption and viral entry processes, suggesting that siRNA against AP2α may enhance HIV-1 replication after the viral entry process of the viral life cycle.

siRNA against AP2α does not affect the RT process of HIV-1 replication

To study the effect of AP2α siRNA on RT process of HIV-1, we performed semi-quantitative analysis of viral DNA in infected
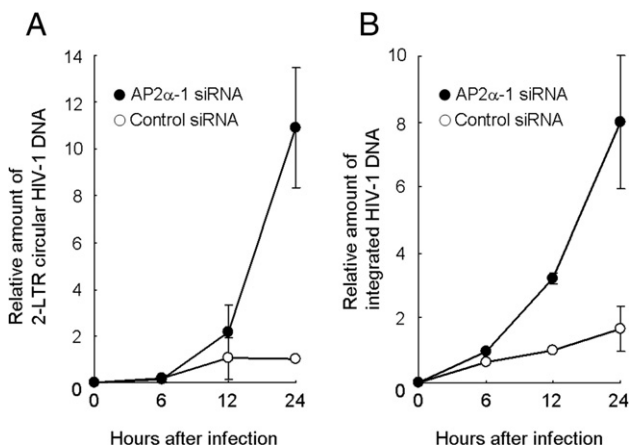

Fig. 5. Viral nuclear translocation as well as viral integration is enhanced by cell transfection with siRNA against AP2α. J111 cells were transfected with AP2α siRNA1 (AP2α-1: solid circle) or control (non-silencing) siRNA (open circle). Forty-eight hours after transfection, the cells were infected with DNase I-treated HIV-1/VSVG (40 ng of p24). Then, Cellular DNA was extracted at 6, 12 and 24 h after infection. Real-time PCR for detecting the 2-LTR circular form of HIV-1 DNA (A) or integrated HIV-1 DNA (B) was performed. The relative amounts of 2-LTR circular form of HIV-1 DNA (A) and integrated proviral DNA (B) were estimated as described in Materials and methods. Data are presented as the means and standard deviations (error bars) of three independent experiments.

cells using real-time PCR. J111 cells were transfected with siRNA and then infected with HIV-1/VSVG. Cellular DNA was then extracted at various times after infection, and we performed real-time PCR to detect early (Fig. 4A) and late (Fig. 4B) products of the viral RT process. The results showed that the kinetics for the reverse transcribed viral DNA production were comparable between cells transfected with siRNA against AP2α and with control siRNA (Fig. 4). The production of viral DNA reached maximum levels at about 6 h after infection and declined thereafter (Fig. 4), indicating that the unintegrated HIV-1 DNA was probably rapidly degraded by cellular nucleases and/or was diluted by growth of cells. In addition, we also examined the viral DNA production in MAGIC5A cells infected with HIV-1/X4 or HIV-1/R5. The results showed that the kinetics for the viral DNA production were comparable between cells transfected with siRNA against AP2α and with control siRNA (Supplementary Fig. S2). These results suggest that the transfection of cells with siRNA against AP2α does not affect the HIV-1 RT process.

The level of nuclear viral DNA as well as integrated proviral DNA is increased in cells transfected with siRNA against AP2α

Next, we studied the nuclear translocation of HIV-1 by measuring the level of the 2-LTR circular form of viral DNA using inverted real-time PCR (Cara et al., 2002; Chang et al., 2005). The results showed that the level of the 2-LTR circular form of HIV-1 DNA was markedly increased at 24 h after infection in cells transfected with siRNA against AP2α compared with control cells (Fig. 5A). In addition, we performed Alu real-time PCR (Bulter et al., 2001; Kameoka et al., 2005) to estimate the level of integrated proviral DNA. The results showed that the level of proviral DNA was significantly increased in cells transfected with siRNA against AP2α compared with control cells (Fig. 5B).
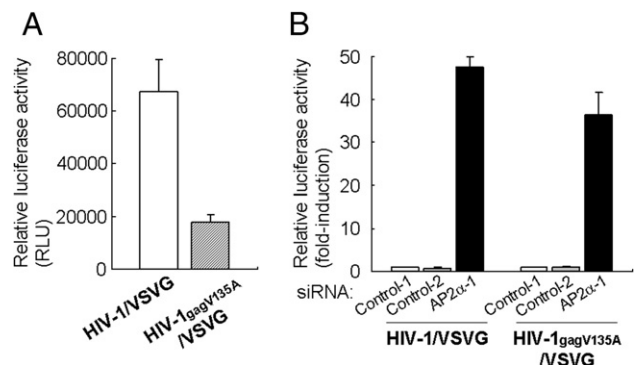

Fig. 6. Interaction between AP-2 and HIV-1 Gag is dispensable for the regulatory function of AP2α in HIV-1 replication in the early phase of the viral life cycle. (A) J111 cells were infected with 40 ng (p24) of HIV-1/VSVG (open bar) or HIV-1gagV135A/VSVG (right hatched bar). Twenty-four hours after infection, luciferase activity in infected cells was measured. (B) J111 cells were transfected with AP2α siRNA1 (AP2α-1: solid bar), control (non-silencing) siRNA (Control-1: open bar) or FLJ10847 siRNA (Control-2: light gray bar). Forty-eight hours after transfection, cells were infected with 40 ng (p24) of HIV-1/VSVG or HIV-1gagV135A/VSVG, as indicated below the panel. Twenty-four hours after infection, luciferase activity in infected cells was measured. Data are shown as fold induction relative to luciferase activity in cells transfected with control siRNA. Data are presented as the means and standard deviations (error bars) of three independent experiments.
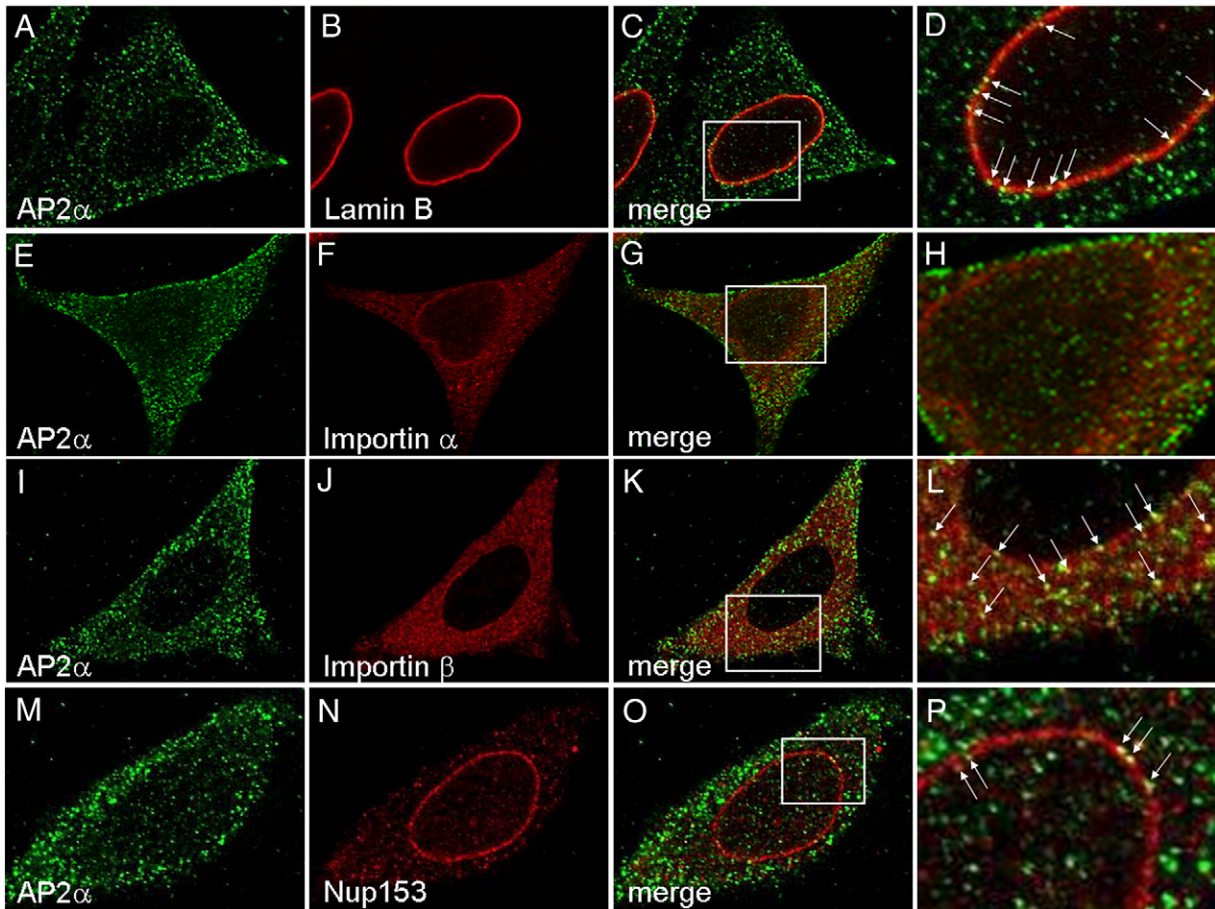
Fig. 7. A subpopulation of AP2α is not only localized in cytoplasm, but also partly co-localized with proteins functioning in the perinuclear region. Permeabilized J111 cells were immunostained using an antibody for AP2α together with those for lamin B (A–D), importin α (E–H), importin β (I–L) or Nup153 (M–P), and fluorescence images were examined by confocal fluorescence microscopy, as described in Materials and methods. Fluorescence images of AP2α (green; A, E, I and M), lamin B (red, B), importin α (red, F), importin β (red, J) and Nup153 (red, N), merged images (C, G, K and O) as well as enlarged areas of merged images (D, H, L and P) are shown. Arrows indicate merged (yellow) signals. Similar images were observed for more than twenty independent experiments.

These results suggest that siRNA against AP2α up-regulates HIV-1 replication in the process of nuclear translocation of viral DNA, and thus, eventually the level of integrated proviral DNA is also increased in cells transfected with siRNA against AP2α.

Interaction between HIV-1 Gag and AP-2 is dispensable for the regulation of HIV-1 replication in the early stage of the viral life cycle by AP2α

AP-2 is reported to play a role in the late phase of the viral life cycle (Batonick et al., 2005; Boge et al., 1998) and also to interact with HIV-1 Gag (Batonick et al., 2005). Amino acid residues Y132 and V135 in HIV-1 Gag polyprotein are important for interacting with AP-2 (Batonick et al., 2005); therefore, we prepared VSVG-pseudotyped mutant viruses possessing Gag but lacking the AP-2 binding site, HIV-1gagY132A/VSVG and HIV-1gagV135A/VSVG, and examined the replication level of those mutant viruses in cells transfected with siRNA against AP2α. Since HIV-1gagY132A/VSVG had no infectivity (data not shown), we used HIV-1gagV135A/VSVG in the following experiments. First, we compared the infectivity of HIV-1gagV135A/VSVG with that of wild-type virus, HIV-1/VSVG.

It can be expected that HIV-1gagV135A/VSVG will show higher infectivity if the interaction between Gag and AP-2 is required for the inhibitory role of AP2α in HIV-1 replication in the early stage of the viral life cycle; however, the results showed that the infectivity of HIV-1gagV135A/VSVG was rather significantly lower than that of wild-type HIV-1 (Fig. 6A). Next, we examined the replication of HIV-1gagV135A/VSVG in J111 cells transfected with siRNA against AP2α. The results showed that cell transfection with siRNA against AP2α significantly enhanced the replication of HIV-1gagV135A/VSVG as well as wild-type virus (Fig. 6B). These results suggest that the interaction between HIV-1 Gag V135 and AP-2 is dispensable for the inhibitory role of AP2α on HIV-1 replication in the early stage of the viral life cycle.

A subpopulation of AP2α is not only localized in the cytoplasm but is also co-localized with proteins that play roles in the perinuclear region

On one hand, AP2α has been found to play a role in the clathrin-dependent endocytosis mechanism at the plasma membrane (Connor and Schmid, 2003; Nakatsu and Ohno, 2003;

Robinson, 2004). On the other hand, our results suggest that AP2α regulates HIV-1 replication in the process of nuclear translocation of viral DNA (Fig. 5), implying that AP2α regulates HIV-1 replication in the cytoplasmic, perinuclear or nuclear regions; therefore, we examined the localization of AP2α by confocal fluorescence microscopy. First, we single-stained cells with the antibody for AP2α, and found that a part of AP2α seemed to be localized in the cytoplasm as well as around the nuclear region, although the majority of AP2α was detected at the plasma membrane (data not shown). The fluorescence signal of AP2α was dramatically decreased after transfecting J111 cells with siRNA against AP2α (Supplementary Fig. S3), indicating that AP2α was specifically immunostained. Therefore, we then double-stained cells with antibodies for AP2α and for the protein functioning in the perinuclear region. The results showed that a subpopulation of AP2α was partly co-localized with nuclear structural protein, lamin B (Fig. 7, panels A–D), nuclear transport receptor, importin β (Fig. 7, panels I–L) and nuclear pore component, Nup153 (Fig. 7, panels M–P). In contrast, the co-localization of AP2α and importin α was not clearly observed (Fig. 7, panels E–H). These results suggest that a subpopulation of AP2α is localized not only in the cytoplasm, but also in the perinuclear region, and possibly regulates HIV-1 replication in the process of nuclear translocation of HIV-1 DNA.

Discussion

In this study, we found a novel inhibitory function of AP2α in HIV-1 replication in the process of nuclear translocation of viral DNA.

siRNA induces the RNAi-mediated specific suppression of target genes in eukaryotic cells (Elbashir et al., 2001). Previously, we studied the level of HIV-1 replication in cells transfected with a series of siRNAs directed against cellular genes, and found that the early stage of HIV-1 replication was significantly enhanced in cells transfected with siRNA against AP2α (Kameoka et al., 2007). In this study, we further studied the mechanism of how AP2α regulates HIV-1 replication. AP2α is a major component of AP-2, and is involved in clathrin-dependent endocytosis as well as the trafficking of molecules from the plasma membrane to endosomes (Connor and Schmid, 2003; Nakatsu and Ohno, 2003; Robinson, 2004). AP-2 is therefore involved in HIV-1 Nef-dependent down-regulation of the CD4 molecule (Chaudhuri et al., 2007; Jin et al., 2005; Rose et al., 2005). In addition, AP-2 plays a role in the internalization of HIV-1 Gag (Batonick et al., 2005) and Env (Boge et al., 1998), eventually leading to the inhibition of virion formation and the release of virion from infected cells.

Our results show that siRNA against AP2α significantly enhanced HIV-1 replication in the early phase of the viral life cycle (Fig. 1), suggesting that AP2α negatively regulates viral replication under normal cell culture conditions. First, we considered that AP2α might play a suppressive role in HIV-1 replication as the functional molecule involved in the clathrin-dependent endocytosis mechanism; however, siRNA against clathrin, which inhibits clathrin-dependent endocytosis (Motley et al., 2003), did not up-regulate HIV-1 replication, in contrast to siRNAs against AP2α (Fig. 1B). In addition, the early stages of

HIV-1 replication, including viral adsorption, viral entry and RT, were not enhanced by the transfection of cells with siRNA against AP2α (Figs. 3 and 4). In stark contrast, the levels of nuclear viral DNA and integrated proviral DNA were significantly up-regulated by transfecting cells with siRNA against AP2α (Fig. 5). These results suggest that AP2α regulates HIV-1 replication independently of its role in the clathrin-dependent endocytosis mechanism in the plasma membrane.

AP-2 is known to interact with HIV-1 Gag and to play a role in the late phase of HIV-1 replication (Batonick et al., 2005). We examined whether this interaction is required to regulate viral replication by AP2α. Our studies using the mutant virus possessing Gag lacking the AP-2 binding site revealed that the interaction between Gag and AP-2 was dispensable for the regulatory function of AP2α for HIV-1 replication (Fig. 6). Thus, it is significant that AP2α regulates HIV-1 replication in the early stage of the viral life cycle by means of a different mechanism from the functions known to regulate HIV-1 replication in the late phase of the viral life cycle.

siRNA against AP2α markedly enhanced the nuclear translocation as well as the integration of HIV-1 DNA (Fig. 5), whereas a subpopulation of AP2α was localized in the cytoplasm and the perinuclear region (Fig. 7). Considering these results, AP2α presumably plays a role in suppressing viral nuclear translocation in the cytoplasm or the perinuclear region, although the detailed mechanism underlying this phenomenon is not understood. One possibility is that AP2α suppresses viral nuclear translocation through the interaction with viral or cellular protein (s) associated with intracellular reverse transcription complex (RTC) or preintegration complex (PIC). Viral proteins, Gag matrix protein (MA), Vpr and integrase, are associated with HIV-1 RTC or PIC, and play roles for the nuclear transport of viral genome (Bouyac-Bertoia et al., 2001; Bukrinsky et al., 1993; Bukrinsky, 2004; Gallay et al., 1997; Heinzinger et al., 1994; von Schwedler et al., 1994). It may be conceivable that AP2α possibly regulates the function of these nucleophilic viral proteins. Another possibility is that AP2α interacts with the cellular protein (s) that plays a role in the process of the nucleocytoplasmic transport of viral components through nuclear pores, and regulates its function. Indeed, our results show that a subpopulation of AP2α was co-localized with Nup153 and importin β (Fig. 7), which are involved in nuclear import of HIV-1 DNA (Bukrinsky, 2004; Gallay et al., 1996, 1997; Popov et al., 1998a,b; Varadarajan et al., 2005, Vodicka et al., 1998), implying that AP2α is involved in the regulation of cellular nuclear import mechanism. Studies on these possibilities are on going in order to elucidate the detailed mechanism(s) of how AP2α regulates HIV-1 replication in the process of nuclear translocation of viral genome.

Materials and methods

siRNA

Two siRNAs directed against AP2α, AP2α siRNA1 (target sequence: 5′-GGUACCGUGUGCUACAGAU-3′) and AP2α siRNA2 (siRNA ID: 5397), were purchased from RNAi Co. (Tokyo, Japan) and Ambion (Austin, Tex.), respectively. AP2α

siRNA1 is equivalent to the siRNA against AP2α included in siRNA mini-library (Kameoka et al., 2007). In addition, the third siRNA against AP2α (AP2α siRNA3, target sequence: 5′-AAGUGGAUGCCUUUCGGGUCA-3′) and siRNA against clathrin (target sequence: 5′-UAAUCCAAUUCGAAGAC-CAAU-3′) (Motley et al., 2003) were prepared using a Silencer™ siRNA Construction kit (Ambion), according to the manufacturer's instructions. As negative controls, siRNA against FLJ10847 (siRNA ID: 140539) and control (non-silencing) siRNA were purchased from Ambion.

Cells

293T, MAGIC-5A [CCR5-expressing MAGI (HeLa-CD4-LTR-β-gal)] (Hachiya et al., 2001; Mochizuki et al., 1999) and J111 cells were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS) (complete medium). It is noteworthy that J111 cells (JCRB0073, Lot # 030395; Japan Health Sciences Foundation, Tokyo, Japan) were originally described as a human acute monocytic leukemia, but many data show this is a subline of HeLa cells (Lavappa, 1978).

siRNA transfection

The cells were transfected with siRNA using HiPerfect transfection reagent (Qiagen, Tokyo, Japan). Briefly, the cells (2–3×10^4 cells/500 μl) were seeded in complete medium in a 24-well plate at 24 h prior to siRNA transfection. The siRNA was mixed with the transfection reagent to form the RNA/reagent complex. The cells were then transfected with siRNA (final 25 nM), according to the manufacturer's protocol.

Viral constructs

The pNL4-3 (Adachi et al., 1986)-based, luciferase reporter viral construct containing the unique recognition sites for BspEI and NotI at the N- and C-terminus, respectively, of the env gene, pNL-Luc-envCT [denoted as pNL-envCT in the reference (Kinomoto et al., 2005b)], was used as X4 HIV-1 (HIV-1/X4). R5 HIV-1 (HIV-1/R5), pNL-Luc-BaLenv, was constructed by replacing the env gene of pNL-Luc-envCT with the corresponding gene fragment of pBa-L (GenBank accession no. AB253432), as follows. Briefly, the env gene of pBa-L was amplified using PfuUltra™ Hotstart DNA Polymerase (Stratagen) and the primers, 5′-TGCtccggaAATGAGAGTGAAGGAGA-3′ and 5′-TGCgcggccgcTTATAGCAAAATCCTTTCC-3′. Lowercase letters indicate the recognition sites for BspE1 (tccgga) and NotI (gcggccgc). After digestion of the PCR product with BspEI and NotI, the env gene fragment of pBa-L was introduced into pNL-Luc-envCT. The luciferase reporter viral construct, pNL-Luc-gagpolCT, containing the unique recognition site for PvuI, ClaI and XbaI at the N-terminus of viral protease, reverse transcriptase and integrase gene, respectively, and for BlpI at the C-terminus of integrase gene, was constructed based on pNL-Luc-ProCT (Kinomoto et al., 2005a) by site-directed mutagenesis. In addition, its Env-deficient derivative, pNL-Luc-E⁻-gagpolCT,

was constructed by introducing a frameshift mutation at the NdeI recognition site in the env gene. pNL-Luc-E⁻-gagY132A and pNL-Luc-E⁻-gagV135A were constructed using pNL-Luc-E⁻-gagpolCT by site-directed mutagenesis. Env-deficient viral constructs, pNL-Luc-E⁻-gagY132A, pNL-Luc-E⁻-gagV135A and pNL-Luc-E⁻R⁺ (Chen et al., 1994; Connor et al., 1995), as well as VSVG-expression vector, pHIT/G (Fouchier et al., 1997), were used to prepare the VSVG-pseudotyped reporter HIV-1 (HIV-1gagY132A/VSVG, HIV-1gagV135A/VSVG and HIV-1/VSVG, respectively). Viral supernatants were prepared by transfecting 293T cells with proviral constructs using FuGENE 6 transfection reagent (Roche), essentially as described (Kameoka et al., 2004). Forty-eight hours after transfection, supernatants were filtered though a 0.45-μm-pore-size filter and stored as aliquots at −85 °C. The viral titer was determined by measuring the concentration of HIV-1 Gag p24 antigen in viral supernatants by enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix Corp., Buffalo, NY).

Viral infectivity assay

Cells were incubated with viral supernatant (40 ng of p24) for 1 h at 37 °C. The viral supernatant was then replaced with complete medium, and the cells were cultured for 24 h at 37 °C. They were then lysed in 100 μl of reporter lysis buffer (Passive Lysis Buffer, Promega), the supernatant (lysate) was clarified at 15,000 rpm for 5 min at 4 °C, and luciferase activity in 10 μl of lysate was determined using the luciferase assay system (Promega). Luciferase activity was measured using Lumat LB 9507 (Berthold Japan, Tokyo, Japan).

Viral adsorption assay

The cells were incubated with viral supernatant (40 ng of p24) for 1 h at 4 °C. After thoroughly washing with cold phosphate-buffered saline (PBS), the cells were lysed in 80 μl of p24 ELISA lysis buffer (supplemented with an ELISA kit to detect p24 antigen). The level of viral adsorption to cells was determined by measuring the concentration of HIV-1 Gag p24 antigen in the cell lysate by ELISA, as described above. Env-deficient HIV-1 virion was prepared by transfecting 293T cells with pNL-Luc-E⁻R⁺, and was used as a negative control.

Viral entry assay

The cells were incubated with viral supernatants (40 ng of p24) for 1 h at 4 °C. After thoroughly washing with cold PBS, the cells were incubated in complete medium for 1 h at 37 °C. They were then trypsinized to remove viruses attached to the cell surface. The cell lysate was then prepared and the amount of p24 antigen in the samples was measured by ELISA, as described above. Env-deficient HIV-1 virion was used as a negative control.

Semi-quantification of reverse transcribed viral DNA

The cells were infected with HIV-1 (40 ng of p24). Prior to infection, the viral supernatant was treated with 1000 U/ml of

RNase-free DNase I (Roche) for 30 min at 25 °C. Cellular DNA was then extracted at various times after infection, using a QIAamp DNA blood mini kit (Qiagen). Real-time PCR was carried out for the semi-quantification of HIV-1 DNA. Briefly, cellular DNA (50–150 ng) was mixed with a 12.5 μl reaction mixture containing 2× *Taq*Man universal PCR master mix (Applied Biosystems), HIV-1-specific primers (300 nM each) and the *Taq*Man probe. HIV-1 specific primers, RS; 5′-AACTAGG-GAACCCACTGCTTAAG-3′ [nucleotide (nt) 500 to 522 of pNL4-3], U5AS; 5′-CGCCACTGCTAGAGATTTTCCAC-3′ (nt 640 to 618), U5S; 5′-CAGACCCTTTTAGTCAGTGTGGAA-3′ (nt 600 to 623) and 5NCAS; 5′-CTCTGGCTTTACTTTCG-CTTTCA-3′ (nt 677 to 655), and the *Taq*Man probes, RU5 probe; 5′-FAM-ACACTACTTGAAGCACTCAAGGCAAGCTTT-TAMRA-3′ (nt 9634 to 9605; corresponding to nt 559 to 530) and U55NC probe; 5′-FAM-TCTCTAGCAGTGGCGCCCGAACA-TAMRA-3′ (nt 626 to 648), were used. Primer pair RS–U5AS and RU5 probe were designed to detect the earliest RT product. Primer pair U5S–5NCAS and U55NC probe were expected to detect only the late RT product synthesized after the second template switch. Real-time PCR was carried out using ABI PRISM 7900HT (Applied Biosystems), according to the manufacturer's instructions. To monitor the efficiency of DNA extraction, β-actin DNA was also amplified using *Taq*Man β-actin control reagents (Applied Biosystems). The plasmid, pNL-Luc-E⁻R⁺, was serially 10-fold diluted and subjected to real-time PCR to make a standard plot, and the copy number of HIV-1 DNA was calculated using SDS software version 2.2.2 (Applied Biosystems). The level of HIV-1 DNA was standardized by dividing the copy number of HIV-1 DNA by that of β-actin to compensate for variations in the quantity or quality of DNA samples, and the results are shown as the copy number of HIV-1 DNA per cell.

Evaluation of the level of viral nuclear translocation

The level of viral nuclear translocation was evaluated by measuring the level of the 2-LTR circular form of viral DNA using inverted PCR, essentially as described previously (Cara et al., 2002; Chang et al., 2005). Real-time nested PCR was carried out as follows. Cellular DNA was subjected to the first PCR using Blend *Taq*^{plus} (Toyobo, Osaka, Japan) and a pair of primers, U3S; 5′-GAGCCCTCAGATGCTGCATATAAG-3′ (nt 9484 to 9507) and U3AS; 5′-GCAGCTCTCGGGCCATGT-GACG-3′ (nt 306 to 285). Then, a 1-μl aliquot of first PCR product was mixed with a 15-μl reaction mixture containing 2× *Taq*Man universal PCR master mix (Applied Biosystems), RS; 5′-AACTAGGGAACCCACTGCTTAAG-3′ (nt 9575 to 9597) (300 nM) and U3AS2; 5′-TCCACAGATCAAGGATATCT-TGTC-3′ (nt 51 to 28) (300 nM) and RU5 probe; 5′-FAM-ACACTACTTGAAGCACTCAAGGCAAGCTTT-TAMRA-3′ (nt 9634 to 9605) (200 nM). Real-time PCR was carried out as described above. The PCR product (2-LTR fragment), amplified by using the primer pair RS-U3AS2, was cloned in pT7Blue T-vector (Novagen). The plasmid containing the 2-LTR fragment was then serially diluted and subjected to real-time PCR to make a standard plot, and the amount of the 2-LTR circular form of HIV-1 DNA was estimated. The level of HIV-1 DNA was standardized by dividing the copy number of HIV-1 DNA by that of β-actin, and the results are shown as the relative amount of the 2-LTR form of HIV-1 DNA.

Evaluation of the level of viral integration

Real-time nested PCR for the semi-quantification of integrated HIV-1 DNA was carried out using the primer specific for Alu DNA repeats, which are randomly distributed, roughly 5000 bp apart, in the chromosome (Jelinek and Schmid, 1982), combined with primers for the HIV-1 genome, essentially as described (Kameoka et al., 2005). Briefly, cellular DNA was subjected to the first PCR using Blend *Taq*^{plus} (Toyobo) and a pair of primers, U3S; 5′-GAGCCCTCAGATGCTGCATATAAG-3′ (nt 9484 to 9507) and Alu primer; 5′-TCCCAGCTACTCGGGAGGC-TGAGG-3′. Then, a 1-μl aliquot of first PCR product was mixed with a 12.5-μl reaction mixture containing 2× *Taq*Man universal PCR master mix (Applied Biosystems), RS; 5′-AAC-TAGGGAACCCACTGCTTAAG-3′ (nt 500 to 522) (300 nM), U5AS; 5′-CGCCACTGCTAGAGATTTTCCAC-3′ (nt 640 to 618) (300 nM) and RU5 probe; 5′-FAM-ACACTACTTGAAG-CACTCAAGGCAAGCTTT-TAMRA-3′ (nt 9634 to 9605; corresponding to nt 559 to 530) (200 nM). Real-time PCR was carried out, as described above. Serially diluted plasmid, pNL-Luc-E⁻R⁺, was subjected to real-time PCR to make a standard plot, and the amount of HIV-1 DNA was estimated, as described above. The level of HIV-1 DNA was standardized by dividing the copy number of HIV-1 DNA by that of β-actin, and the results are shown as the relative amount of HIV-1 proviral DNA.

Immunoblotting

Cells were lysed in sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0.003% bromophenol blue]. Samples were then separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat milk in PBS, the blots were immunostained with anti-AP2α (adaptin α) monoclonal antibody (#610502; BD Biosciences, San Jose, CA), anti-clathrin heavy chain monoclonal antibody (#61500; BD Biosciences) or anti-α-tubulin monoclonal antibody (#T5168; Sigma, Saint Louis, MO). After incubation of the samples with peroxidase-labeled secondary antibodies, the immuno-complex was visualized using ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

Confocal fluorescence microscopy

For immunofluorescence analysis, J111 cells were seeded in a Lab-Tek™ II chamber slide (Nalge Nunc International). Twenty-four hours later, the cells were fixed with 4% paraformaldehyde for 15 min at 4 °C, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. The samples were then immunostained with an antibody for AP2α (#610502; BD Biosciences), followed by an Alexa 488-conjugated secondary antibody. For double staining, the samples were further stained

with an antibody for lamin B (sc-6217; Santa Cruz Biotechnology, Santa Cruz, CA), importin α (sc-6917, Santa Cruz Biotechnology), importin β (sc-1863, Santa Cruz Biotechnology) or Nup153 (#BP4506; Acris Antibody GmbH, Hiddenhausen, Germany), followed by Alexa 594- or Cy3-conjugated secondary antibody. Fluorescence images of the cells were observed using a laser-scanning confocal microscope, Fluoview FV1000 (Olympus, Tokyo, Japan) with a 60× oil immersion objective (NA1.35). Fluorochromes were excited using an argon laser for Alexa 488, and a helium/neon laser for Alexa 594 and Cy3. Image analysis was carried out using standard functions of the FV10-ASW viewer software version 1.6 (Olympus).

Evaluation of HIV-1 Nef-dependent CD4 down-regulation

The *nef* gene of pNL4-3 was cloned in pcDNA3.1 (Invitrogen) to generate a Nef-expression vector, pcDNA-Nef. MAGIC5A cells were transfected with clathrin siRNA, AP2α siRNA1 or control (non-silencing) siRNA. Forty-eight hours after siRNA transfection, cells were further transfected with pcDNA-Nef or pcDNA3.1 (2 μg) using the Nucleofector-II device (Amaxa Biosystems, Nattermannallee 1, Germany; Cell Line Nucleofector Kit R and program I-13). Forty-eight hours after DNA transfection, cells were harvested and immunostained with phycoerythrin (PE)-conjugated anti-CD4 monoclonal antibody (#347327, BD Biosciences). As a negative control, cells were incubated with PE-conjugated mouse IgG2b (#33805X, BD Biosciences). Then, samples were subjected to flow cytometry using FACSCalibur (BD Biosciences) with CellQuest software (BD Biosciences).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.11.033.

References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59, 284–291.

Batonick, M., Favre, M., Boge, M., Spearman, P., Höning, S., Thali, M., 2005. Interaction of HIV-1 gag with the clathrin-associated adaptor AP-2. Virology 342, 190–200.

Boge, M., Wyss, S., Bonifacino, J.S., Thali, M., 1998. A membrane-proximal tyrosine-based signal mediates internalization of the HIV-1 envelope glycoprotein via interaction with the AP-2 clathrin adaptor. J. Biol. Chem. 273, 15773–15778.

Bouyac-Bertoia, M., Dvorin, J.D., Fouchier, R.A.M., Jenkins, Y., Meyer, B.E., Wu, L.I., Emerman, M., Malim, M.H., 2001. HIV-1 infection requires a functional integrase NLS. Mol. Cell 7, 1025–1035.

Bukrinsky, M., 2004. A hard way to the nucleus. Mol. Med. 10, 1–5.

Bukrinsky, M.I., Haggerty, S., Dempsey, M.P., Sharova, N., Adzhubei, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., Stevenson, M., 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature 365, 666–669.

Bulter, S.L., Hansen, M.S.T., Bushman, F.D., 2001. A quantitative assay for HIV DNA integration in vivo. Nat. Med. 7, 631–634.

Cara, A., Vargas, J., Keller, M., Jones, S., Mosoian, A., Gurtman, A., Cohen, A., Parkas, V., Wallach, F., Chusid, E., Gelman, I.H., Klotman, M.E., 2002. Circular viral DNA and anomalous junction sequence in PBMC of HIV-infected individuals with no detectable plasma HIV RNA. Virology 292, 1–5.

Chang, T.L., Vargas, J., DelPortillo, A., Klotmanm, M.E., 2005. Dual role of α-defensin-1 in anti-HIV-1 innate immunity. J. Clin. Invest. 115, 765–773.

Chaudhuri, R., Lindwasser, O.W., Smith, W.J., Hurley, J.H., Bonifacino, J.S., 2007. Downregulation of CD4 by human immunodeficiency virus type 1 Nef is dependent on clathrin and involves direct interaction of Nef with the AP2 clathrin adaptor. J. Virol. 81, 3877–3890.

Chen, B.K., Saksela, K., Andino, R., Baltimore, D., 1994. Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by super-infection of nonproductively infected cell lines with recombinant luciferase-encoding viruses. J. Virol. 68, 654–660.

Connor, R.I., Chen, B.K., Choe, S., Landau, N.R., 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mono-nuclear phagocytes. Virology 206, 935–944.

Connor, S.D., Schmid, S.L., 2003. Regulated portals of entry into the cells. Nature 422, 37–44.

Cullen, B.R., 2003. Nuclear mRNA export: insights from virology. Trends Biochem. Sci. 28, 419–424.

Daecke, J., Fackler, O.T., Dittmar, M.T., Kräusslich, H.-G., 2005. Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. J. Virol. 79, 1581–1594.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., Landau, N.R., 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381, 661–666.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., Paxton, W.A., 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381, 667–673.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediated RNA interference in cultured mammalian cells. Nature 411, 494–498.

Feng, Y., Broder, C.C., Kennedy, P.E., Berger, E.A., 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane G-protein-coupled receptor. Science 272, 872–877.

Fouchier, R.A.M., Meyer, B.E., Simon, J.H.M., Fischer, U., Malim, M.H., 1997. HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. EMBO J. 16, 4531–4539.

Gallay, P., Stitt, V., Mundy, C., Oettinger, M., Trono, D., 1996. Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. J. Virol. 70, 1027–1032.

Gallay, P., Hope, T., Chin, D., Trono, D., 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc. Natl. Acad. Sci. U. S. A. 94, 9825–9830.

Hachiya, A., Aizawa-Matsuoka, S., Tanaka, M., Takahashi, Y., Ida, S., Gatanaga, H., Hirabayashi, Y., Kojima, A., Tatsumi, M., Oka, S., 2001. Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 using CCR5-expressing HeLa/CD4+ cell clone 1–10 (MAGIC-5). Antimicrob. Agents Chemother. 45, 495–501.

Heinzinger, N.K., Bukrinsky, M.I., Haggerty, S.A., Ragland, A.M., Kewalramani, V., Lee, M.-A., Gendelman, H.E., Ratner, L., Stevenson, M., Emerman, M., 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. U. S. A. 91, 7311–7315.

Jelinek, W.R., Schmid, C.W., 1982. Repetitive sequences in eukaryotic DNA and their expression. Ann. Rev. Biochem. 51, 813–844.

Jin, Y.-J., Cai, C., Zhang, Y.X., Zhang, H.-T., Hirst, J.A., Burakoff, S.J., 2005. HIV Nef-mediated CD4 down-regulation is adaptor protein complex 2 dependent. J. Immunol. 175, 3157–3164.

Kameoka, M., Kitagawa, Y., Utachee, P., Jinnopat, P., Dhepakson, P., Isarangkura-na-ayuthaya, P., Tokunaga, K., Sato, H., Komano, J., Yamamoto, N., Oguchi, S., Natori, Y., Ikuta, K., 2007. Identification of the suppressive factors for human immunodeficiency virus type-1 replication using the siRNA mini-library directed against host cellular genes. Biochem. Biophys. Res. Commun. 359, 729–734.

Kameoka, M., Nukuzuma, S., Itaya, A., Tanaka, Y., Ota, K., Ikuta, K., Yoshihara, K., 2004. RNA interference directed against poly(ADP-ribose) polymerase 1 efficiently suppresses human immunodeficiency virus type 1 replication in human cells. J. Virol. 78, 8931–8934.

Kameoka, M., Nukuzuma, S., Itaya, A., Tanaka, Y., Ota, K., Inada, Y., Ikuta, K., Yoshihara, K., 2005. Poly(ADP-ribose) polymerase-1 is required for integration of the human immunodeficiency virus type 1 genome near centromeric alphoid DNA in human and murine cells. Biochem. Biophys. Res. Commun. 334, 412–417.

Kinomoto, M., Appiah-Opong, R., Brandful, J.A.M., Yokoyama, M., Nii-Trebi, N., Ugly-Kwame, E., Sato, H., Ofori-Adjei, D., Kurata, T., Barre-Sinoussi, F., Sata, T., Tokunaga, K., 2005a. HIV-1 proteases from drug-naïve West African patients are differentially less susceptible to protease inhibitors. Clin. Infect. Dis. 41, 243–251.

Kinomoto, M., Yokoyama, M., Sato, H., Kojima, A., Kurata, T., Ikuta, K., Sata, T., Tokunaga, K., 2005b. Amino acid 36 in the human immunodeficiency virus type 1 gp41 ectodomain controls fusogenic activity: implications for the molecular mechanism of viral escape from a fusion inhibitor. J. Virol. 79, 5996–6004.

Lavappa, K.S., 1978. Survey of ATCC stocks of human cell line for HeLa contamination. In Vitro 14, 469–475.

Mochizuki, N., Otsuka, N., Matsuo, K., Shiino, T., Kojima, A., Kurata, T., Sakai, K., Yamamoto, N., Isomura, S., Dhole, T.N., Takabe, Y., Matsuda, M., Tatsumi, M., 1999. An infectious DNA clone of HIV type 1 subtype C. AIDS Res. Hum. Retrovir. 15, 1321–1324.

Motley, A., Bright, N.A., Seaman, M.N.J., Robinson, M.S., 2003. Clathrin-mediated endocytosis in AP-2-depleted cells. J. Cell Biol. 162, 909–918.

Nakatsu, F., Ohno, H., 2003. Adaptor protein complexes as the key regulators of protein sorting in the post-Golgi network. Cell Struct. Funct. 28, 419–429.

Popov, S., Rexach, M., Ratner, L., Blobel, G., Bukrinsky, M., 1998a. Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. J. Biol. Chem. 273, 13347–13352.

Popov, S., Rexach, M., Zybarth, G., Reiling, N., Lee, M.-A., Ratner, L., Lane, C.M., Moore, M.S., Blobel, G., Bukrinsky, M., 1998b. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. EMBO J. 17, 909–917.

Rice, A.P., Kimata, J.T., 2006. Cellular cofactors and HIV-1 infection in vivo. Future Virol. 1, 337–347.

Robinson, M.S., 2004. Adaptable adaptors for coated vesicles. Trends Cell Biol. 14, 167–174.

Rose, J.J., Janvier, K., Chandrasekhar, S., Sekaly, R.P., Bonifacino, J.S., Venkatesan, S., 2005. CD4 down-regulation by HIV-1 and simian immunodeficiency virus (SIV) Nef proteins involves both internalization and intracellular retention mechanisms. J. Biol. Chem. 280, 7413–7426.

Sokolskaja, E., Luban, J., 2006. Cyclophilin, TRIM5, and innate immunity to HIV-1. Curr. Opin. Microbiol. 9, 404–408.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., Sodroski, J., 2004. The cytoplasmic body component TRIM5α restricts HIV-1 infection in old world monkeys. Nature 427, 848–853.

Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., Saigo, K., 2004. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. 32, 936–948.

Varadarajan, P., Mahalingam, S., Liu, P., Ng, S.B.H., Gandotra, S., Dorairajoo, D.S.K., Balasundaram, D., 2005. The functionally conserved nucleoporins Nup124p from fission yeast and the human Nup153 mediate nuclear import and activity of the Tf1 retrotransposon and HIV-1 Vpr. Mol. Biol. Cell 6, 1823–1838.

Vodicka, M.A., Koepp, D.M., Silver, P.A., Emerman, M., 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev. 12, 175–185.

von Schwedler, U., Kornbluth, R.S., Trono, D., 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc. Natl. Acad. Sci. U. S. A. 91, 6992–6996.

Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H., Jones, K.A., 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell 92, 451–462.

Zheng, Y.-H., Yu, H.-F., Peterlin, B.M., 2003. Human p32 protein relieves a post-transcriptional block to HIV replication in murine cells. Nat. Cell Biol. 5, 611–618.

Zheng, Y.-H., Lovsin, N., Peterlin, B.M., 2005. Newly identified host factors modulate HIV replication. Immunol. Lett. 15, 225–234.